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Reticuloendothelial system blockade by PEG-oligo(amino acid) block copolymers: A strategy for functional tuning of nanomedicine pharmacokinetics

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Our transient and selective stealth coating strategy is expected to (i) improve the efficacy of gene therapy drugs, (ii) reduce the dose required to obtain therapeutic outcomes, and (iii) decrease dose-related toxicities, which ultimately lead to the reduction of the medical cost of nanomedicines.

A single biggest issue of systemically injected nanomedicines is nonspecific elimination by the liver sinusoidal wall cells, which substantially decrease the delivery efficiency at diseased sites. We addressed this issue by stealth coating of liver sinusoidal wall using single- or two-armed poly(ethylene glycol) (PEG)-conjugated oligo(l-lysine)(OligoLys). PEG-OligoLys selectively coated to the liver sinusoidal wall, leaving the other tissue endothelium uncoated and, thus, accessible to the nanomedicine delivery. Interestingly, 2-arm PEG-OligoLys uncoated the sinusoidal wall within hours and cleared to the bile, while 1-arm PEG-OligoLys persisted at the wall. Such transient and selective stealth coating of sinusoidal wall by two-arm PEG-OligoLys was effective in preventing the sinusoidal clearance of nonviral and viral gene vectors, representatives of synthetically-engineered and nature-derived nanomedicines, respectively, thereby boosting their gene transduction at the diseased sites.

Biomaterials/Biomedical engineering-related

RES blockade　PEG coating to liver　Two-arm PEG-peptide　Bile clearance　Retargeting nanomedicine

Nanotheragnostics (NTGs) have been extensively used for the efficient delivery of therapeutic and diagnostic agents into diseased tissues. However, when NTGs are systemically applied into a living body, NTGs are rapidly cleared and metabolized by reticuloendothelial system organs, particularly the liver. In the liver, the sinusoidal wall (endothelial cells plus Kupffer cells) has a high endocytic activity to clear NTGs actively from the bloodstream owing to their recognition by several types of scavenger receptors (SRs). The sinusoidal wall capture both artificially engineered and nature-derived NTGs, such as viral gene vectors, limiting their delivery efficiency to target disease sites.

To address liver sinusoidal wall-mediated clearance, surface stealth modification of NTGs, e.g., by poly(ethylene glycol) (PEG), has been extensively studied to permit their stable circulation in the bloodstream for hours to days. However, most of the NTGs are still subjected to sinusoidal clearance even after surface stealth modification. Thus, a combination of other strategies that decrease the sinusoidal wall clearance of NTGs is highly demanded. Among them, modulation of host-tissue clearance pathways is a promising option. Preinjection of SR ligands, such as fucoidan, polyinosinic acid (poly-I), and dextran sulfate (DS), or decoy nano/microparticles is widely used to oversaturate the endocytic functions of the sinusoidal wall. However, this strategy has two major problems. First, agents used for SR saturation inhibit only specific pathways of clearance, depending on the SR type or clearance site that they target, even though the liver sinusoids have diverse elimination pathways. Even a single NTG can be recognized by several SRs, thus simultaneous inhibition of several elimination pathways is preferred. Second, SR saturation strategy often raises toxicity issues including inflammation induced by fucoidan or poly-I and anticoagulation induced by DS.

To circumvent the above-mentioned issues, this study focused on transient and selective stealth coating of the liver sinusoidal wall, using precisely designed PEG-conjugated positively charged oligopeptide. In contrast to the SR saturation, PEG coating to the sinusoidal wall would be effective for the simultaneous inhibition of several clearance pathways. The PEG coating should be transient and selective to the liver sinusoidal wall to avoid toxicity issues. This was achieved by using oligo(L-lysine) (OligoLys) conjugated two-armed PEG (two-arm-PEG-OligoLys) for anchoring PEG to the sinusoidal wall. PEG-OligoLys avoided the nonspecific binding of OligoLys to other tissue endothelium, presumably via PEG stealth property, with preserved attachment to liver wall, which have high binding affinity to positively charged oligopeptides because of the abundance of negatively charged receptors. The clearance behavior of PEG-OligoLys was successfully modulated by optimizing the PEG configuration, two-arm-PEG-OligoLys displayed transient coating to the sinusoidal wall, followed by gradual biliary clearance, while one-arm-PEG-OligoLys displayed long-term attachment. Subsequently, transient and selective stealth coating of the sinusoidal wall by two-arm-PEG-OligoLys was found to be effective in avoiding the sinusoidal clearance of nonviral and viral gene vectors, offering an increased gene transfer efficiency to their intrinsic target tissues via their redirection from the liver sinusoid wall.

Accumulation of NTGs to the liver scavenger sinusoidal wall is the single biggest issue to the clinical translation because it not only impedes the dose availability to target tissues but also can raise toxicity concerns. To overcome this issue, the current study was designed. The main purpose is to develop potential and safe sinusoidal wall stealth coating agent using PEG-Oligopeptide to suppress the liver uptake, simultaneously improving the circulatory time of NTGs for precise biodistribution to extra-hepatic tissues.

All real-time observations in the liver and earlobe were performed using an A1R in vivo confocal laser scanning microscopy (IVCLSM) (Nikon Corp., Japan). Earlobe was fixed beneath the coverslip to observe the connective tissue blood vessels. The liver was surgically exposed and glued directly to the cover glass for the observation of sinusoids. Dye-labeled OligoLys with or without PEG was injected at a similar dose. The intensity at the sinusoidal wall and lumen of earlobe vessels was quantified over time.

Colon adenocarcinoma tumor-bearing mice were injected with luciferase (Luc)-expressing plasmid (p)DNA nanomachine, before and after two-arm-PEG-OligoLys coating. Tumors were collected, homogenized to obtain lysate for the Luc measurement.

Mice were injected with Luc encoding adeno-associated virus serotype 8 (AAV8)

before and after two-armed PEG coating. Three weeks later, the liver, heart, and skeletal muscle were excised and homogenized to obtain lysate for the Luc expression analysis.

An ideal coating agent should satisfy two following criteria. First, the coating should be selective to liver sinusoidal walls because coating to other tissue vessel walls throughout the body would not only impede the delivery efficiency of NTGs to target tissues but also cause adverse toxicological effects. Second, the coating should be transient because long-term coating may impair normal functions of the liver. To meet these criteria, we have precisely designed a coating agent, two-arm-PEG-OligoLys, which demonstrated selective and transient coating at the sinusoidal wall.

Selective stealth coating of the sinusoidal wall using one- and two-PEG-OligoLys: Non-PEGylated OligoLys was attached to the vessel walls of the earlobe, a representative connective tissue, immediately after injection. On the contrary, both one- and two-arm-PEG-OligoLys did not attach to the earlobe vessel wall. Thus, the attachment of OligoLys to the vessel walls of connective tissue was successfully avoided by PEGylation of OligoLys, presumably due to PEG steric repulsion property.

Transient stealth coating of the sinusoidal wall using two-PEG-OligoLys: The amount of two-arm-PEG-OligoLys at the sinusoidal wall gradually decreased and became undetectable at 6 hours (**Fig. 1b**), whereas one-arm-PEG-OligoLys remained almost similar amount even at 9 hours after injection (**Fig. 1a**). Closer observation revealed that two-arm-PEG-OligoLys was progressively accumulated to the space between the hepatocytes (bile canaliculi) at 3 hours, whereas one-arm-PEG-OligoLys was undetectable to that space even at 9 hours. This result indicates that two-arm-PEG-OLys was able to be cleared from the sinusoidal wall to the bile in several hours, thus no chronic accumulation toxicity can be anticipated. In this way, a precise molecular design was essential to obtain transient coating. Finally, two-armed PEG coating was subsequently applied to boost the delivery efficacy of gene therapy drugs.
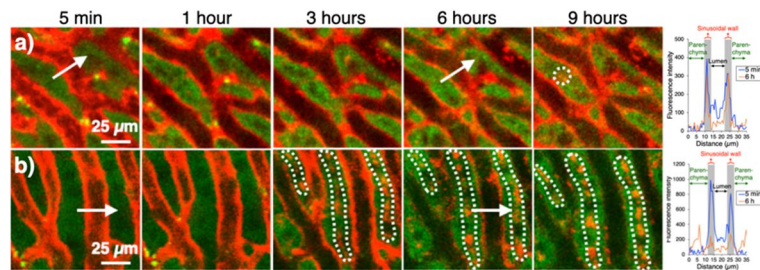
Fig. 1. Coating of one-arm-PEG-OligoLys **(a)** and two-arm-PEG-OligoLys **(b)** to the liver sinusoidal wall and their bile clearance observed by IVCLSM. PEG–OligoLys, liver parenchymal autofluorescence, location of bile canaliculi are encircled in white dotted lines.

Relocating viral gene carriers from the sinusoidal wall to their target tissues: AAV8 is widely used in gene therapy, targets the heart and skeletal muscle. However, liver sinusoidal clearance seriously impedes its ability to reach its target tissues. When AAV8 was administered after prior coating of two-armed PEG to the liver sinusoidal wall, the transfer of AAV8 to the liver was suppressed, and as a result, the gene transfer efficiency into the heart and skeletal muscle was improved by 2 to 4 times (**Fig. 2a**).

Redirecting pDNA-equipped nanomachine to the tumor: We extended our strategy to virus-free gene delivery systems, offer economically cheap and safe gene therapy. In the absence of two-arm-PEG coating, DNA nanomachine was captured by the sinusoidal wall, even though PEGylated. On the contrary, two-armed PEG coating to the sinusoidal wall before the nanomachine injection, effectively suppressed the nanomachine clearance by the sinusoidal wall, resulting in 10-fold improvement in DNA transfer efficiency to colon cancer (**Fig. 2b**).
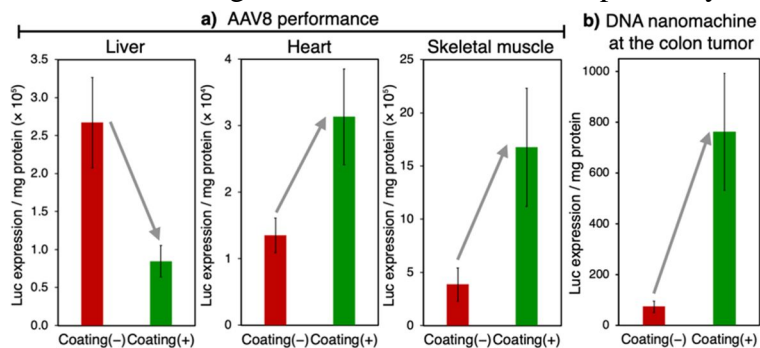
Fig. 2. Systemic performance of gene therapy drugs with (+) and without (−) 2-armed PEG coating. **(a)** AAV8 retargeting from liver to heart and skeletal muscle, and **(b)** Relocation of DNA nanomachine to the tumor.

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